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Micromolar concentrations of Al^{3+} induce phase separation, aggregation and dye release in phosphatidylserine-containing lipid vesicles

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The interaction of Al^{3+}, Cd^{2+} and Mn^{2+} with phosphatidylserine-containing lipid vesicles was studied. Phase separation of vesicles was investigated by monitoring fluorescence quenching of the phospholipid analogue 1-palmitoyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminocaproyl)phosphatidylcholine (C_6-NBD-PC). Aggregation was determined by turbidimetry and leakage of vesicles content during fusion was monitored by the fluorescence of released 6-carboxyfluorescein. Al^{3+} demonstrated quenching at less than 30 μmol/l with a maximum effect at 100 μmol/l. Al^{3+}-induced aggregation and dye release from the lipid vesicles were observed in the same concentration range. The effect of Cd^{2+} and Mn^{2+} on quenching was much less pronounced and could only be demonstrated in the 0.1–1 mmol/l range. Increasing amounts of phosphatidylcholine or phosphatidylethanolamine in the vesicles decreased both Al^{3+}-induced quenching and aggregation, wheras cholesterol only slightly increased aggregation without affecting quenching.

Introduction

Neurotoxic effects of aluminum, cadmium and manganese have been documented [1–3]. The biochemical basis of these effects is not well established. Aluminum has been considered by several authors to be the toxic causal agent involved in renal dialysis dementia [4] and there is some evidence that aluminum could also be involved in the pathogenesis of senile dementia [3,5]. Recently, it was demonstrated that Al^{3+} increases the permeability of the blood-brain barrier [6]; it has been reported that the transport of choline in red blood cells is affected in senile dementia [7]. On the other hand, ther is also evidence that aluminum, cadmium and manganese strongly inhibit choline transport in erythrocytes and synaptosomes [8,9].

To explore the mechanism of the neurotoxicity oof these cations further we therefore decided to study their effect on phospholipid model membranes.

Materials and Methods

Bovine brain phosphatidylserine, egg yolk phosphatidylcholine, egg yolk phosphatidylethanolamine and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO); C_6-NBD-PC was purchased from Avanti Polar Lipids (Birmingham, Al) and 6-carboxyfluorescein from Eastman Kodak (Rochester, NY).

Small unilamellar vesicles of various lipid compositions were obtained in 0.12 mol/l NaCl, 0.02 mol/l Tris-HCl (pH 7.4) by three successive ultrasonications of 60 s at 75 W with a Branson Sonifier B 12 (Branson Co., Soest, The Netherlands)

Abbreviations: NBD, 4-nitrobenz-2-oxa-1,3-diazole; C_6-NBD-PC, 1-palmitoyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminocaproyl)phosphatidylcholine.

under nitrogen flux at 4°C from a multilamellar vesicles suspension (5 mg phospholipids in 2 ml) containing 5 mol% C_6-NBD-PC [10]. The concentration of liposome was 32 μmol/l in all quenching experiments (except when noted).

Small unilamellar vesicles for aggregation experiments were obtained in the same way and the concentration in all experiments was 50 μmol/l.

The liposomes used for the leakage of 6-carboxyfluorescein (fusion experiments) were obtained by ultrasonication (300 s at 75 W) of multilamellar vesicles (10 mg/ml) in a buffer, 100 mmol 6-carboxyfluorescein/NaOH (pH 7.4) and separated by passage through an equilibrated Sephadex G-75 column as previously described [11]. Liposomes were diluted to obtain a final concentration of 30 μmol of phospholipid. 6-Carboxyfluorescein was used without further purification.

The NBD or 6-carboxyfluorescein fluorescence was continuously monitored either with an SLM 4800 Aminco Spectrofluorometer (SLM-Aminco, Urbana, IL) equipped with a HP 7470A plotter or with a Farrand Mark I Spectrofluorometer (Farrand Optical Co., New York, NY) equipped with a chart recorder. NBD and 6-carboxyfluorescein were excited at 475 and 490 nm and monitored at 530 and 550 nm, respectively. The aggregation was monitored by the turbidity changes of the unilamellar vesicles suspension at 400 nm using a Beckman Model 24 Spectrophotometer (Beckman Fullerton CA).

Quenching of NBD was calculated according to the formula:

$$Q = \frac{I_0 - I_{max} - \Delta I_c}{I_0 - \Delta I_c}$$

where I_0 represents the fluorescence intensity of the vesicle population at the beginning of the experiment, I_{max}, the maximal decrease of fluorescence intensity and ΔI_c, the decrease in fluorescence intensity due to sample dilution when adding an aqueous volume of 220 μl to the liposome suspension (2000 μl).

Results

Quenching of NBD

The effect of increasing concentrations of calcium, manganese, cadmium and aluminum on the quenching of C_6-NBD-PC (5 mol%) incorporated into phosphatidylserine liposomes is demonstrated in Fig. 1. Plateau values for quenching used for Fig. 1–4 were reached after less than 4 min of incubation, independently of the concentration of the cation or of the composition of the membranes studied. Approximate values for half-maximal effects (ED_{50}) for these ions were 50, 500, 700 and 900 μmol/l for Al^{3+}, Cd^{2+}, Mn^{2+} and Ca^{2+}, respectively.

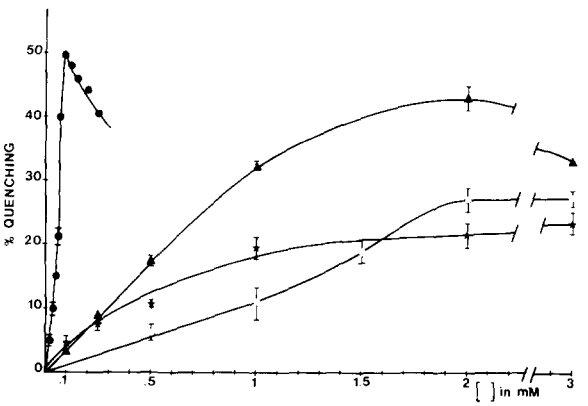

Fig. 1. Al^{3+}-, Cd^{2+}-, Mn^{2+}- and Ca^{2+}-induced fluorescence quenching in phosphatidylserine vesicles as a function of cation concentration. 65 nmol of C_6-NBD-PC/phosphatidylserine (5:95) were suspended in 2 ml of buffer and the cations were added to the final concentration indicated, ●, Al^{3+}; ▲, Cd^{2+}; ★, Mn^{2+}; ○, Ca^{2+}. Each point is the mean of three to four experiments for the divalent cations and of at least six experiments for Al^{3+}.

Fig. 2 shows that quenching of fluorescence

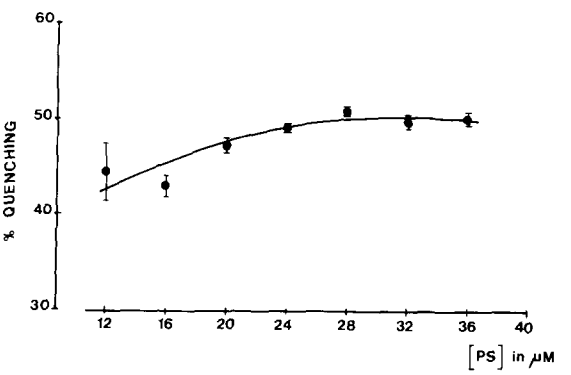

Fig. 2. Al^{3+} (100 μmol/l)-induced fluorescence quenching in phosphatidylserine vesicles as a function of vesicles concentration. Each point is the mean (±S.D.) of three experiments.

depends on the concentration of Al^{3+} and not on the ratio of Al^{3+} to phosphatidylserine.

It is important to note that increasing the concentration of Al^{3+} did not affect the fluorescence of C_6-NBD-PC when incorporated into phosphatidylcholine liposomes (data not shown). These findings exclude any direct effect of the cation on the probe used.

When liposomes were incubated simultaneously with aluminum and calcium, the sequence of addition of the cation determined the effects observed. As can be seen from Table I, pre-incubation with 1 mmol/l Ca^{2+} partially inhibited the effect of low Al^{3+} concentration.

Fig. 3a shows that the quenching effect of Al^{3+} could be partially inhibited by citrate, indicating chelation of Al^{3+} by the tricarboxylic acid [12,13]. Aluminum-induced quenching of fluorescence was also altered by increasing amounts of phosphatidylcholine in the liposomes. However, the addition of cholesterol did not modify the effect of the cation (see Fig. 3b).

Release of 6-carboxyfluorescein

The release of 6-carboxyfluorescein from unilamellar phosphatidylserine liposomes by increasing concentrations of Al^{3+} was studied by measur-

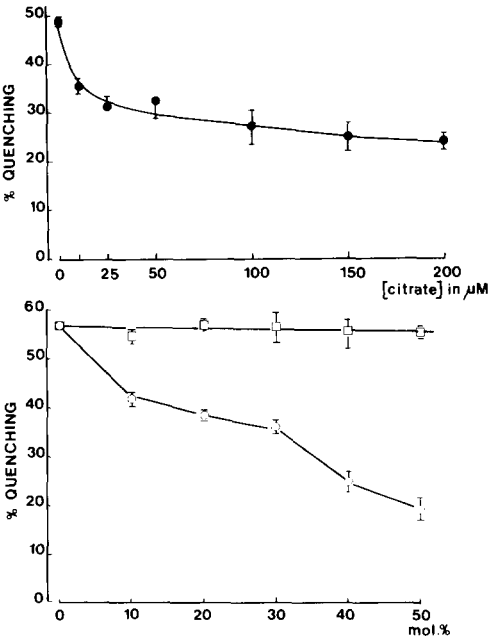

Fig. 3. (a) Inhibition of Al^{3+} (100 μmol/l)-induced NBD fluorescence quenching by citrate as a function of citrate concentration. (b) Aluminum-induced NBD quenching in mixed phosphatidylserine/phosphatidylcholine (○) and in mixed phosphatidylserine/cholesterol vesicles (□). Liposomes consisting of phosphatidylserine and phosphatidylcholine (○) or cholesterol (□) and containing 5 mol% C_6-NBD-PC were incubated in the presence of 100 μmol/l Al^{3+}. The extent of quenching was determined after 4 min when quenching reached a plateau value.

TABLE I

EFFECT OF THE SEQUENCE OF ADDITION OF Al^{3+} AND Ca^{2+} ON QUENCHING

Time between addition of 1st and 2nd cation was 2 min. The concentrations are in mmol and the Q_1, Q_2 and Q_{tot} values represent the quenching due to the first ion added, the quenching due to the second ion added after 2 min and the total quenching, respectively. Results are the mean ± S.E. of six to ten experiments.

		Q_1	Q_2	Q_{tot}
Ca →	Al			
1	0.025	9.8 ±0.3	1.4±0.8	11.2
1	0.050	9.9 ±0.3	11.9±1.9	21.8
1	0.075	9.8 ±0.3	16.0±1.2	25.8
1	0.100	9.8 ±0.3	54.0±2.2	63.8
Al →	Ca			
0.025	1	6.6 ±1.30	9.9±2.4	16.5
0.050	1	17.20±1.10	11.7±1.4	29.0
0.075	1	38.90±1.30	10.4±1.6	49.3
0.100	1	50.20±0.40	9.8±1.8	60.0

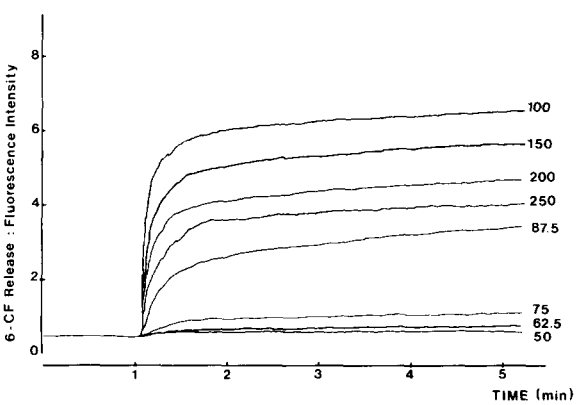

Fig. 4. Extent of 6-carboxyfluorescein (6-CF) release from sonicated phosphatidylserine vesicles (30 μmol/l) after addition of Al^{3+} at the various concentrations indicated (in μmol/l). Maximum fluorescence intensity obtained by the addition of 0.1% of Triton X-100 to a vesicle suspension was set at 9 arbitrary units before experiments.

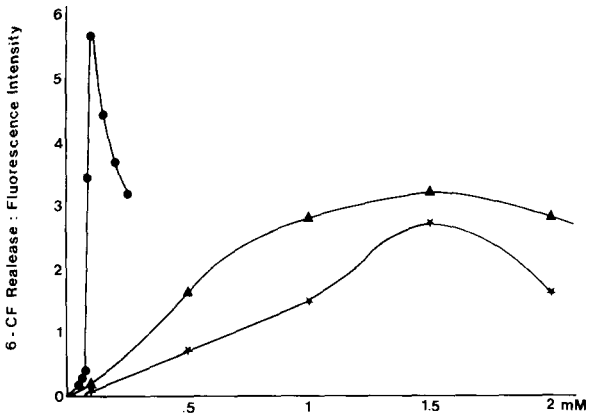

Fig. 5. Al^{3+}-, Cd^{2+}- and Mn^{2+}-induced 6-carboxyfluorescein (6-CF) release from phosphatidylserine vesicles after 4 min as a function of cation concentration. ●, Al^{3+}; ▲, Cd^{2+}; ★, Mn^{2+}.

ing changes in fluorescence intensity, following the addition of the cation.

Fig. 4 demonstrates that the effect was concentration-dependent and that dequenching of the probe was apparent at concentrations of Al^{3+} less than 75 μmol/l. When plateau values were plotted against the concentrations of the three cations used, the data presented in Fig. 5 was obtained. It is apparent from these data that Al^{3+} has a pronounced effect on the release of the probe from the liposomes, quite distinguishable from the effect seen with Cd^{2+} and Mn^{2+}. These effects are quantitatively similar to those observed in the quenching experiments (Fig. 1). Approximate ED_{50} values were 50, 500 and 750 μmol/l for Al^{3+}, Cd^{2+} and Mn^{2+}, respectively.

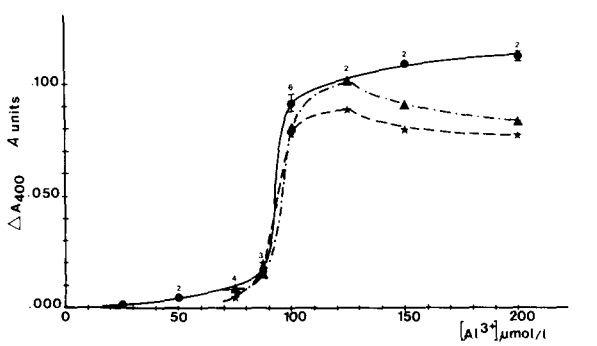

Fig. 6. Turbidity changes after 4 min with vesicles (50 μmol/l) of different phospholipid composition vs. Al^{3+} concentration. ●, phosphatidylserine; ▲, phosphatidylserine/phosphatidylethanolamine (4:1), ★, phosphatidylserine/phosphatidylcholine (4:1). (The number of experiments is shown in the figure.)

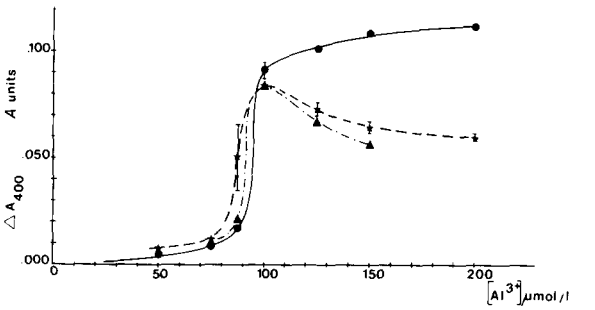

Fig. 7. Turbidity changes after 4 min with vesicles (50 μmol/l) of different phospholipid composition vs. Al^{3+} concentration. ●, phosphatidylserine; ▲, phosphatidylserine/cholesterol (4:1); ★, phosphatidylserine/cholesterol (3:2).

Aggregation of liposomes

Aggregation of pure unilamellar phosphatidylserine liposomes or liposomes containing variable amounts of phosphatidylethanolamine, phosphatidylcholine or cholesterol was studied at increasing concentrations of aluminum. As can be seen from Figs. 6 and 7, phosphatidylethanolamine or phosphatidylcholine did not affect the threshold concentration of Al^{3+} for aggregation, but significantly reduced maximum aggregation. A molar ratio of phosphatidylserine/cholesterol of either 8:2 or 6:4 did, however, slightly shift the concentration threshold for Al^{3+}-induced aggregation towards lower concentrations of the cation and also significantly decreased maximum aggregation.

Discussion

The data presented here clearly demonstrate that the three cations used alter the physical state of phosphatidylserine-containing membranes. In all the experiments carried out (quenching, aggregation and 6-carboxyfluorescein release), aluminum always showed the most powerful effect at concentrations at least 10-times lower than any of the other cations. ED_{50} ratios were 10, 15 and 18 for Al^{3+}/Cd^{2+}, Al^{3+}/Mn^{2+} and Al^{3+}/Ca^{2+}, respectively. The ED_{50} value obtained for calcium is close to the concentratioan reported by others

studying the effect of this cation on fluorescence quenching of C_6-NBD-PC in phosphatidylserine liposomes [10].

It is evident from Fig. 1 that aluminum has a dramatic effect on NBD fluorescence quenching which is maximum at 100 μM Al^{3+}. Since addition of Al^{3+} to C_6-NBD-PC/phosphatidylcholine vesicles does not affect the fluorescence intensity and, since NBD fluorescence is unaffected by the membrane microviscosity [10], the quenching of NBD-PC observed here could well reflect lipid phase separation. Location of the fluorescent moiety excludes the possibility that quenching is due to aggregation.

Increasing concentrations of aluminum demonstrated a bell-shaped effect on quenching with a maximum at 100 μM. It is possible that presentation to the membrane of high amounts of the trivalent ion induces rigidification of the bilayer, thus hindering any further phase separation. This would require Al^{3+}-induced phase separation of phosphatidylserine lipids to be a slower process than membrane rigidification.

Quenching of C_6-NBD-PC always reached plateau values after a maximum of 3–4 min of incubation with Al^{3+}, although approx. 95% of this value is reached in less than 1 min. This observation contrasts with the time delay reported by others when studying the time-course of Ca^{2+}-induced quenching of NBD-PC in phosphatidylserine vesicles or in mixed phosphatidyserine/phosphatidylcholine vesicles [10].

It has also been reported that increasing concentrations of cholesterol decreased the quenching effect of Ca^{2+} [10], while in our experiments with Al^{3+}, an effect of cholesterol on cation-induced quenching was not observed. The initial fluorescence levels were, however, lowered by cholesterol. The differences between the data reported with Ca^{2+} and our findings with Al^{3+} could be due to particular affinity of this ion for phosphatidylserine lipids.

When phosphatidylserine vesicles are preincubated with 1 mM Ca^{2+} and then exposed to low concentrations of aluminum (25–75 μM), quenching is less than in the absence of calcium (Table I).

However, with increasing concentrations of Al^{3+} (100–125 μM), the increments in quenching are higher, suggesting a steep dependence of the quenching phenomenon on aluminum when calcium is present.

Our findings suggest that both ions mutually interact with the membrane to promote cooperative Al^{3+}-induced phase separation. Concentrations of aluminum required to produce these effects are 10–12-times lower than the concentration of calcium. Al^{3+} would therefore bind to phosphatidylserine membranes with an affinity approx. one order of magnitude higher than that of Ca.

Several studies [12,13] have demonstrated that citrate chelates aluminum with a high association constant (10^8 $mol^{-1} \cdot l$). Partial inhibition by citrate of the quenching effect of Al^{3+} observed in our experiments (Fig. 3a) suggests high affinity of Al^{3+} for phosphatidylserine lipids.

6-Carboxyfluorescein release also reach plateau values after short incubation times with either Al^{3+}, Cd^{2+} or Mn^{2+} (Figs. 4 and 5). As in our quenching experiments, it could be seen in Figs. 4 and 5 that the optimal concentration of Al^{3+} inducing maximum release of 6-carboxyfluorescein was approx. 100 μM. At a higher aluminum concentration, massive and rapid binding could stabilize the membranes or the fused aggregates, making them less leaky. This could explain the peculiar concentration dependence observed here.

The absorbance monitoring the Al^{3+}-induced vesicles aggregation reached plateau values within 1 min. The presence of either phosphatidylethanolamine, phosphatidylcholine or cholesterol seems to affect the aggregation process at higher Al^{3+} concentrations.

A partial increase in light transmission was also observed at a high concentration of Al^{3+} when liposomes contained either 20 mol% of phosphatidylethanolamine or phosphatidylcholine (Fig. 6) or 20 and 40% of cholesterol. Secondary formation of superagregates induced by fusion of smaller vesicles could be the explanation for the decrease in absorbance at high Al^{3+} concentrations observed with mixed liposomes.

Finally, it is remarkable that quenching, aggregation and release were all observed at the same concentration of aluminum, whereas binding [15,16], quenching [10,14], aggregation [14,16–17], fusion (6-carboxyfluorescein [14–17], [14–17], or Tb-dipicolinic acid (14,17–18)) and lipid intermixing [18,20] reported with Ca^{2+} in phosphati-

dylserine vesicles was observed over a wider concentration range (1–4 mmol/l).

Lanthanum is, according to our knowledge the only trivalent cation studied so far for its effect on phosphatidylserine vesicles. The effects reported are similar to those observed here with aluminum [21,22].

The concentrations of Al^{3+}, Cd^{2+} and Mn^{2+} required to obtain half-maximum effects (ED_{50}) are close to the ED_{50} values reported for inhibition of choline transport in erythrocytes and brain synaptosomes [8,9]. This might suggest that at least some of the toxic effect of Al^{3+} on biological processes could be caused by or associated with a direct interaction of aluminum with phospholipid membrane constituents.

This paper presents evidence that Al^{3+}, in concentrations only slightly higher than those normally encountered in biological fluids [23], profoundly alters the physical state of phosphatidylserine-containing membranes. We believe that these findings constitute a possible rational basis for explaining some of the toxic effects of aluminum at the molecular level.

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